

MATERIAL INSPECTION AND RECEIVING REPORT

Form Approved
OMB No. 0704-0248

Public reporting burden for this collection of information is estimated to average 35 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0248), Washington, DC 20503.

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO EITHER OF THESE ADDRESSES.

1. PROC. INSTRUMENT IDEN. (CONTRACT)				(ORDER NO)	6. INVOICE NO./DATE	7. PAGE	8. ACCEPTANCE POINT	
N00014-95-C-0209				667	1	3	D	
2. SHIPMENT NO.	3. DATE SHIPPED	4. B/L	5. DISCOUNT TERMS		NET			
MIC012	9/2/97	TCN	NA		DCMAO ATLANTA 805 WALKER STREET MARIETTA, GA 300060-2789			
9. PRIME CONTRACTOR				10. ADMINISTERED BY		CODE S1103A		
MICROBIAL INSIGHTS INC. 2340 STOCK CREEK BLVD ROCKFORD, TN 37853-3044				11. SHIPPED FROM (if other than 9)		12. PAYMENT WILL BE MADE BY		
SEE BLOCK 9				CODE FOB	D	CODE SC1020		
13. SHIPPED TO				14. MARKED FOR		CODE ONR 331		
PROGRAM OFFICER (ATTN: KEITH WARD) OFFICE OF NAVAL RESEARCH 800 NORTH QUINCY STREET ARLINGTON, VA 22217-5660				PROGRAM OFFICER (ATTN: KEITH WARD) OFFICE OF NAVAL RESEARCH 800 NORTH QUINCY STREET ARLINGTON, VA 22217-5660		ONR 331		
15. ITEM NO	16. STOCK/PART NO. (Indicate number of shipping containers - type of container - container number)			DESCRIPTION	17. QUANTITY SHIP/REC'D.	18. UNIT	19. UNIT PRICE	20. AMOUNT
0001 A M	Final Report			Final Report	Final Report	LO	100,000.	7,494.00
<div style="border: 1px solid black; padding: 5px; display: inline-block;"> DISTRIBUTION STATEMENT <u>B</u> Approved for public release Distribution Unlabeled </div>								
21. CONTRACT QUALITY ASSURANCE								
A. ORIGIN			B. DESTINATION					
<input type="checkbox"/> CQA <input type="checkbox"/> ACCEPTANCE of listed items has been made by me or under my supervision and they conform to contract, except as noted herein or on supporting documents.			<input type="checkbox"/> CQA <input type="checkbox"/> ACCEPTANCE of listed items has been made by me or under my supervision and they conform to contract, except as noted herein or on supporting documents.					
DATE	SIGNATURE OF AUTH GOVT REP		DATE	SIGNATURE OF AUTH GOVT REP				
TYPED NAME AND OFFICE			TYPED NAME AND TITLE					
22. RECEIVER'S USE								
Quantities shown in column 17 were received in apparent good condition except as noted.								
DATE RECEIVED				SIGNATURE OF AUTH GOVT REP				
TYPED NAME AND OFFICE								
* If quantity received by the Government is the same as quantity shipped, indicate by (✓) mark, if different, enter actual quantity received below quantity shipped and encircle.								

23. CONTRACTOR USE ONLY

DTIC QUALITY INSPECTED 3

Application of molecular techniques to define mechanisms of microbially influenced corrosion of stainless steel and copper in marine systems (N 00014-95-C-0299)

James A. Coyer, David C. White

Final Report

August 1997

Bacteria play a major role in the processes of biofouling and biocorrosion as members of biofilms and regulators of nutrient cycling in these systems. In these capacities, bacterial communities change temporally and spatially due to consortial interactions. Some bacterial species may facilitate or inhibit development of metabolic capacity of other bacterial species in the biofilm. To fully understand the processes of consortial interactions, therefore, the identity and activity of component species in a consortium must be resolved at the relevant temporal and spatial scales.

The objectives of our research were to resolve the temporal and spatial patterns of N₂-fixing and sulfate-reducing bacteria within a bacterial consortium. Specifically,

1. Develop *in situ* hybridization techniques (*in situ* PCR) with fluorescent probes to detect the temporal abundance and spatial distribution of N₂-fixing, and sulfate-reducing bacteria in biofilms
2. Map the spatial distribution of the bacteria using congruently *in situ* hybridization techniques and confocal microscopy

Several approaches were directed toward Objective 1. First, a biofilm was simulated by smearing fixed bacterial cells onto a glass microscope slide. The first experiment used immuno *in situ* techniques, whereby the anti-nitrogenase antibody was applied to a slide smeared with: a) *V. natriegens* cells that were harvested when fixing N₂, b) *V. natriegens* cells that were not fixing N₂, and c) the sulfate-reducing bacterium (SRB), *D. vulgaris*. A strong fluorescent signal was present in the N₂-fixing cells, but not in the non-N₂-fixing cells or the SRB. The same assemblage of cells also was probed with EUB, a probe specific for a 18 bp conserved region of the SSU in all eubacteria. The 18 bp/rhodamine-coupled probe produced a strong signal in both *V. natriegens* and *D. vulgaris*. Thus, we are able to introduce antibody and DNA probes into whole bacterial cells fixed onto glass slides.

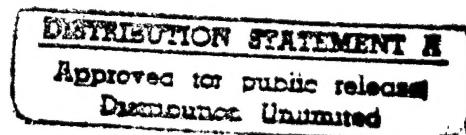
Secondly, we used direct *in situ* PCR techniques to detect naturally and PCR-amplified nucleic acid targets within bacterial cells fixed onto glass slides. Detection of naturally-amplified targets (rRNA) produced equivocal results as the SRB-specific probe (SRB-385; Amann et al. 1992. Appl. Environ. Micro. 58:614-23) did not always produce a signal in the SRB *D. vulgaris*. Direct *in situ* PCR detection of PCR-amplified targets, however, regularly produced signals. Primers (L1, G1) were used to both amplify and label (with DIG) the spacer region between the SSU and LSU in fixed cells of *Vibrio* and the amplified target was detected by a primary antibody (anti-DIG) and a colorimetric assay.

Third, we used direct and indirect PCR techniques to detect low-copy number targets (protein-coding genes) within bacterial cells in solution or fixed onto glass slides. In the direct method, PCR primers (Hyd1, Hyd5) were used to both amplify and label (with DIG), a 1 kb region of the [NiFe] hydrogenase gene in fixed cells, both in solution and on slides, of *Desulfovibrio vulgaris*. The amplified region was detected with a primary antibody (anti-DIG) and a colorimetric assay, as well as with the primary antibody coupled to the fluorochrome, rhodamine.

In the indirect method, the primers (Hyd 2, Hyd 5) were used to amplify the 1 kb region of the hydrogenase gene which subsequently was detected with a rhodamine-labeled probe (Hyd 1). The advantage of the indirect over the direct approach to *in situ* PCR hybridization is that the former results in far fewer false positives.

We also used a two-species system consisting of N₂-fixing cells and SRB. N₂-fixing can be detected by the immuno assay described above and all fixed cells (N₂-fixing and SRB) can be detected with a DNA stain (propidium

19970909 084



iodide). Each type of cell can be distinguished by the different emission wavelengths from the fluor-coupled nitrogenase antibody and the propidium iodide: cells with both signals (N_2 -fixing) emit a different wavelength than cells with only propidium iodide (SRB). We have successfully applied this technique to a simulated biofilm consisting of N_2 and SRB cells.

Applying immuno and direct and indirect PCR techniques to natural biofilms grown in special flowcells (Palmer and Caldwell. 1995. *J. Microbiol. Methods* 24:171-182), however, proved problematic. As the volume of the flowcells ($250 \mu\text{L}$) was much larger than the volume used for PCR on simulated biofilms ($(25 \mu\text{L})$, the subsequent 10-fold increase in the PCR reaction mix lead to negative results. Furthermore, investigation revealed that application of fixatives and the various reagents and washes necessary for *in situ* PCR hybridization invariably distorted or removed the biofilm. The flowcell design has been modified and the new design currently is being evaluated.

In summary, detection of specific types of bacteria within a biofilm may be best accomplished using fluorescent *in situ* hybridization (FISH) techniques employing probes of fluorochrome-coupled antibodies, rather than using either direct or indirect *in situ* PCR hybridization techniques to amplify regions of DNA. Immuno *in situ* techniques are faster, and because of the specific antibody-antigen reaction, produce very little background and/or false-positive signals. As long as antibodies are available for proteins indicative of specific physiological traits (nitrogenase for N_2 -fixing, hydrogenase for sulfate-reducing) expressed by members of a biofilm consortium, clear "yes-no" answers can be formed.

In situ PCR techniques, however, frequently produce confusing results because of high levels of background and/or false-positive signals, both of which develop in spite of stringent reaction conditions. Indeed, the stringent reaction conditions may be so specific as to preclude development of a "universal" protocol for the ever-changing assemblage of species in a consortium. The expense of purifying proteins for antibody production is not trivial, but once developed, the highly specific antibody-antigen reaction will lead to a more rigorous and unequivocal approach to mapping component species in a biofilm consortium.